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Extended B cell phenotype in patients with myalgic encephalomyelitis/chronic fatigue syndrome: a cross-sectional study

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Summary

Myalgic encephalomyelitis/chronic fatigue syndrome (ME/CFS) is a heterogeneous condition of unknown aetiology characterized by multiple symptoms including fatigue, post-exertional malaise and cognitive impairment, lasting for at least 6 months. Recently, two clinical trials of B cell depletion therapy with rituximab (anti-CD20) reported convincing improvement in symptoms. A possible but undefined role for B cells has therefore been proposed. Studies of the relative percentages of B cell subsets in patients with ME/CFS have not revealed any reproducible differences from healthy controls (HC). In order to explore whether more subtle alterations in B cell subsets related to B cell differentiation exist in ME/CFS patients we used flow cytometry to immunophenotype $CD19^+$ B cells. The panel utilized immunoglobulin (Ig)D, CD27 and CD38 (classical B cell subsets) together with additional markers. A total of 38 patients fulfilling Canadian, Centre for Disease Control and Fukuda ME/CFS criteria and 32 age- and sex-matched HC were included. We found no difference in percentages of classical subsets between ME/CFS patients and HC. However, we observed an increase in frequency ($P < 0{\cdot}01$) and expression (MFI; $P = 0{\cdot}03$) of CD24 on total B cells, confined to IgD^+ subsets. Within memory subsets, a higher frequency of $CD21^+CD38^-$ B cells (>20%) was associated with the presence of ME/CFS [odds ratio: 3·47 (1·15–10·46); $P = 0{\cdot}03$] compared with HC, and there was a negative correlation with disease duration. In conclusion, we identified possible changes in B cell phenotype in patients with ME/CFS. These may reflect altered B cell function and, if confirmed in other patient cohorts, could provide a platform for studies based on clinical course or responsiveness to rituximab therapy.

Keywords: B cells, chronic fatigue syndrome, flow cytometry, human, myalgic encephalomyelitis

Introduction

Myalgic encephalomyelitis/chronic fatigue syndrome (ME/CFS) is characterized by multiple symptoms, including fatigue, post-exertional malaise and cognitive impairment, which last for at least 6 months. The diagnosis of ME/CFS is made currently in patients with prolonged fatigue having many of the additional symptoms in the Canadian, Center for Disease Control (CDC) and Fukuda diagnostic criteria [1–3]. The majority of patients with ME/CFS have a history of documented viral or other infections prior to onset of their illness [4,5]. This has been suggested to underlie some of the immunological abnormalities often described in patients with ME/CFS [6–9]. However, strong evidence for persistent or chronic infection with microorganisms is lacking in the majority of patients. The consistency of results of biomedical research, which has been applied to the study of ME/CFS, coupled with the wide range of physical, neurocognitive and autonomic symptoms reported, have seriously hampered attempts to understand pathophysiological pathways in ME/CFS.

Differences from healthy controls (HC) have, however, been suggested by some studies of the immune system. Several groups have described a decrease in cytotoxic activity

of natural killer (NK) and T cells, predominantly by the reduction of granzyme or perforin levels [10–13], similar to which is also associated commonly with herpes virus infections [14]. In contrast, Curriu *et al.* found an increase of NKp46 (natural cytotoxicity on NK cells) and CD69 (activation marker) expression on NK cells [15]. Genomic polymorphisms of killer cell immunoglobulin-like receptors (KIRs) have also been described with an excess of KIR3DS1 combined with increased levels of ligand-free KIRDL1, which may hamper the recognition of target structures of pathogens by NK cells [16].

Cytokine levels in serum and cerebrospinal fluid of ME/CFS patients, compared with those in HC, have also been inconsistent [17–22]. As with immune cell phenotype and function, this may be due to differences in laboratory methodologies, patient selection, phase of illness as well as the timing of sampling.

Rather than comparing to HC, Hickie *et al.* evaluated cytokine production prospectively (post-infection) in patients with proven Eptein–Barr virus (EBV) who had developed ME/CFS compared with those who did not develop ME/CFS after an EBV infection, but no significant differences were found [23]. Interestingly, a study by Brodrick *et al.* of adolescent patients developing ME/CFS following infectious mononucleosis (IM) compared with controls who recovered normally showed an increase in interleukin (IL)-8 and a decrease in IL-5 and IL-23 cytokines [24]. The IL-8 and IL-5 pattern is also seen in asthma and in B cell chronic lymphocytic leukaemia (B-CLL) [25,26]. The most significant difference was the level of IL-23, which was lower in ME/CFS patients. IL-23 is essential for the full and sustained differentiation of the inflammatory T helper type 17 (Th17) T cell subset [27].

Focus on the possible involvement of B cells in expression of ME/CFS was boosted after both investigator- and patient-reported improvement following B cell depletion therapy with single treatment and maintenance therapy using rituximab [28,29]. Response to rituximab-based therapy in autoimmunity is associated usually with the presence of pathogenic autoantibodies. In ME/CFS, there are a number of reports of autoantibodies [30–33]. Anti-muscarinic and anti-adrenergic antibodies have been described in two studies in some ME/CFS patients [33,34]. Studies of B cell phenotypes in ME/CFS have sometimes shown differences from HC [35–37]. For example, Klimas *et al.* described an elevated proportion of CD20- and CD21-positive B cells, confirmed by Tirelli *et al.* in 1994, and also an increase in absolute numbers of CD19- and CD5-positive B cells [38,39]. More recently, Bradley *et al.* showed significant increases in transitional and naive B cell subsets compared with HC, but in contrast, naive B cells were reportedly decreased and memory B cells increased in another study by Brenu *et al.* [11,40].

In order to provide a possible platform for studies of B cell function in patients with ME/CFS we have used a more extensive panel of phenotypical markers to define B cell subsets. The aim, therefore, was to investigate the expression of additional phenotypical markers associated with B cell maturation, differentiation and activation. Many different combinations of surface markers have been used to identify stages of B cell differentiation in malignancy, immunodeficiency and autoimmunity: specifically, CD5, CD23, CD24, CD38, CD27 and immunoglobulin (Ig)D [40–45].

In this study we have used the mature B cell (Bm)1–Bm5 classification (IgD/CD38) to identify transitional B cells, naive B cells, memory populations including post-germinal-centre cells and plasmablasts, as well as IgD/CD27 for naive, switched and preswitched memory B cells [42,46]. Additional markers such as B cell activating factor (BAFF)-receptor (BAFF-R), CD5, CD21 and CD24 were also included. This approach has been used in patients with Sjögren's syndrome [42], but not yet in ME/CFS.

Levels of B cell products including BAFF, soluble CD23 (sCD23), as a measure of acquisition on CD27 phenotype, serum free light chains (SFLC) as a measure of plasmablast activity and serum immunoglobulins (IgA, IgG and IgM) were also measured.

Materials and methods

Patients and HC

Patients diagnosed with ME/CFS fulfilling consensus criteria (Canadian, CDC and Fukuda) were selected for the study by S. B. (Royal London Hospital of Integrated Medicine) and A. B. (St Helier Hospital NHS Trust). Clinical assessments were then performed to assess patients' eligibility for study inclusion. Patients were informed verbally about the study and additionally given information sheets with written informed consent. Medical history (disease duration, symptoms and co-morbidities) was recorded for the purpose of the study. At visit, after study inclusion, data from the Health Assessment and Depression Score (HADS) were collected. Patients with confirmed autoimmune disease or receiving immunosuppression were excluded from the study, as well as those who had a history of depression (HADS > 21), which was not linked to ME/CFS. The severity of chronic fatigue symptoms were assessed using both Bell (*The Doctor's Guide to Chronic Fatigue Syndrome*; 1995) and Cox and Findley (*Severe and very Severe Patients with Chronic Fatigue Syndrome: Perceived Outcome Following and Inpatient Programme*; 2000) scales at visit. HC were age- and sex-matched individuals recruited from hospital staff, and volunteers among family and friends of patients without evidence of ME/CFS on the basis of completed diagnostic criteria questionnaires. Whole blood and serum samples were available from 38 ME/CFS patients (range 18–71) and 32 age- and sex-

Table 1. Patient demographics and serology: healthy controls (HC) *versus* myalgic encephalomyelitis/chronic fatigue syndrome (ME/CFS) patients.

	HC (n = 32)	ME/CFS patients (n = 38)	P-value
Mean age in years (range)	39 (16–74)	40 (18–71)	0·79†
Percentage female	59·4	64·9	n.a.
Serology	Median (range)		P value
BAFF (ng/ml)	0·3 (0·2–1·7)	0·3 (0·2–0·9)	0·39
sCD23 (pg/ml)	2479 (927·2–6679)	1946 (740·7–5663)	0·48
κ SFLC (mg/l)	12·6 (6·0–39·3)	12·5 (6·4–28·2)	0·85
λ SFLC (mg/l)	9·9 (5·9–25·2)	9·4 (2·0–107·7)	0·86
κ/λ SFLC ratio	1·2 (0·7–2·9)	1·3 (0·1–2·2)	0·73
IgA (g/l)	2·5 (0·8–5·1)	2·6 (0·8–5·9)	0·54
IgG (g/l)	10·8 (6·8–18·7)	12·8 (7·5–22·4)	0·02*
IgM (g/l)	0·9 (0·4–2·0)	1·2 (0·3–4·9)	0·07

Normal reference ranges: B cell activating factor (BAFF) (<1·8 ng/ml); soluble CD23 (sCD23) (1235–5024 pg/ml); κ serum-free light chains (κSFLC) (3·3–19·4 mg/l); λSFLC (5·7–26·3 mg/l); κ/λ SFLC (0·3–1·7); immunoglobulin (Ig)A (0·9–5–0 g/l); IgG (6·1–16·2 g/l); IgM (0·4–2·4 g/l). †Mann–Whitney *U*-test was used to determine differences between HC and ME/CFS patients; n.a. = not applicable; *significant ($P < 0·05$).

matched HC (range 16–74); their demographics are presented in Table 1.

This study has been approved by the NRES Committee London–City Road and Hampstead Research ethics Committee (REC reference: 14/LO/0388).

B cell immunophenotyping

Three ml of whole blood was washed twice in phosphate-buffered saline (PBS) and cells divided over two tubes and stained for 20 min with fluorescent conjugates of CD19, CD38 (Biolegend, San Diego, CA, USA) IgD, CD21, IgM, CD5, CD23 (BD Biosciences, San Jose, CA, USA) CD27, CD24 and BAFF-R (eBioscience, San Diego, CA). Combinations are shown in Table 2. Erythrocytes were lysed with BD PharmLyse™ lysing solution, washed twice in PBS and resuspended in 2% paraformaldehyde to fix the cells. Finally, cells were washed and resuspended in PBS and acquired within 24 h on a BD LSR Fortessa™. Gating strategies for IgD/CD27 and IgD/CD38 are shown in Fig. 2. Compensation beads (BD Biosciences) were used to optimize fluorescence compensation settings for multi-colour flow cytometric analysis.

Measurement of soluble factors associated with B cell survival and differentiation

Commercial enzyme-linked immunosorbent assay (ELISA) kits were used to measure serum sCD23 and BAFF/BLyS levels (R&D Systems Europe Ltd, Abingdon, UK); the normal range and upper limit of normal range given by the manufacturers was 1235–5024 pg/ml and <1·8 ng/ml, respectively. Both κ and λ SFLC (normal ranges 3·3–19·4 mg/l and 5·7–26·3 mg/l, respectively) and serum total immunoglobulins IgA (normal range 0·9–5·0 g/l), IgG (normal range 6·1–16·2 g/l) and IgM (normal range 0·4–2·4 g/l) were measured by Binding Site (Birmingham, UK) using their in-house methods and reference values.

Statistics

Comparisons of levels of serum factors and B cell phenotypes (both frequency and expression) between patients and HC were made using non-parametric tests (Mann–Whitney *U*-test), and linear regression (Pearson's correlation coefficient) using GraphPad Prism version 6 (GraphPad, San Diego, USA, USA) with a significance level of 5%. Odds ratios [95% confidence interval (CI)] were calculated from the 3 × 2 contingency table based on tertiles of the distribution.

Results

Patients and serology

Demographics of ME/CFS patients and HC and measures of serum analytes are shown in Table 1. Patients with respiratory or other infections at visit were excluded from the study. The majority of patients were of moderate severity, as assessed by their referring clinician (S. B. and A. B.).

Table 2. Antibody panel for whole blood staining.

Tube							
1	CD19	IgD	CD38	CD27	CD5	CD21	CD24
	PE/Cy7	BV421	PerCP/Cy5·5	APC	FITC	PE	APC-eFluor® 780
2	CD19	IgD	CD38	CD27	CD23	BAFF-R	IgM
	PE/Cy7	BV421	PerCP/Cy5·5	APC-eFluor® 780	FITC	PE	APC

Ig = immunoglobulin; PE/Cy7 = phycoerythrin/cyanin 7; APC = allophycocyanin; FITC = fluorescein isothiocyanate; BAFF = B cell activating factor; PerCp = peridinin chlorophyll.

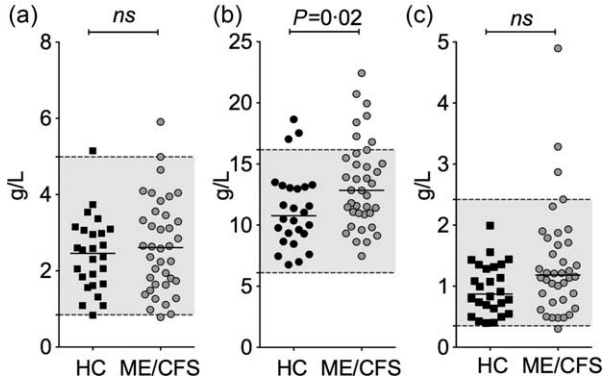

Fig. 1. Serum total immunoglobulin levels in patients with myalgic encephalomyelitis/chronic fatigue syndrome (ME/CFS) ($n = 38$) and age- and sex-matched healthy controls (HC) ($n = 26$). Concentrations (g/l) of serum immunoglobulins are shown for immunoglobulin (Ig)A (a), IgG (b) and IgM (c). Each symbol represents one individual: solid line represents median, shaded areas show reference ranges given by Binding Site (UK) and *P*-values are shown (Mann–Whitney *U*-test; n.s. = not significant).

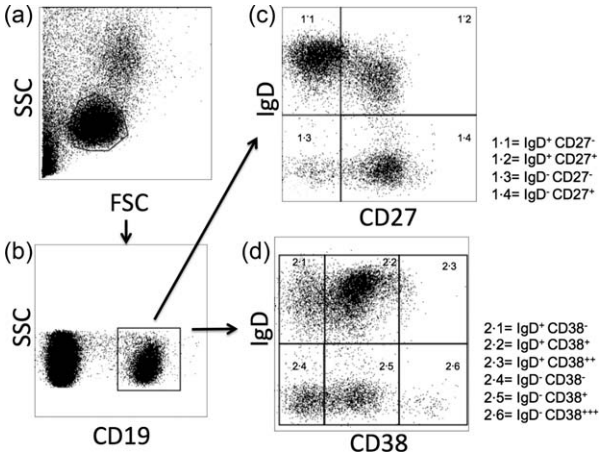

Fig. 2. B cell gating strategy. Representative plots (using whole blood from a healthy control) showing (a) lymphocytes selected based on side-scatter *versus* forward-scatter in which (b) B cells expressing CD19 were then selected. In (c) and (d), respectively, immunoglobulin (Ig)D/CD27 and IgD/CD38 B cell subpopulations are shown.

There was no significant difference between HC and ME/CFS patients with respect to age and sex (Table 1).

When serum levels of BAFF, sCD23 and SFLC (ratio κ/λ) were compared, no significant differences were found between ME/CFS patients and HC (Table 1). As shown in Fig. 1, levels of total serum IgA and IgM were also not different from HC (Fig. 1a,c), but IgG levels in ME/CFS patients were raised significantly when compared with HC ($P = 0{\cdot}02$; Mann–Whitney *U*-test) and seven of 35 had levels above the upper limit of normal range (Fig. 1b).

Classical B cell subsets as defined using IgD, CD27 and CD38

Representative plots of the classical B cell subsets defined by IgD/CD27 (Fig. 2c) and IgD/CD38 (Fig. 2d) are shown in Fig. 2. As shown in Table 3, no significant differences were found between frequencies (% $CD19^+$ B cells) of all nine B cell subsets and of plasmablasts between ME/CFS patients and HC.

Naive and memory B cell populations assessed by IgD/CD38 were confirmed by the additional usage of the memory marker CD27 (Supporting information, Fig. S1). Transitional (IgD^+CD38^{++}) and naive (IgD^+CD38^+) B cells were low for CD27, while IgD^+ memory (IgD^+CD38^-), post-GC (IgD^-CD38^+), resting memory (IgD^-CD38^-) and plasmablasts (IgD^-CD38^{+++}) consisted of predominantly $CD27^+$ cells (Supporting information, Fig. S1a). No significant differences were found between HC and patients with ME/CFS.

Expression of other markers within IgD/CD38 B cell subsets

Using IgD and CD38, we thus distinguished between five B cell subsets and plasmablasts and found no difference between HC and ME/CFS patients (Table 3). The frequencies of additional B cell markers (BAFF-R, CD21, CD23, CD5, IgM and CD24) within each subset defined by IgD/CD38 were then determined.

The frequency of BAFF-R, a principal receptor required for BAFF-mediated B cell survival in humans mainly of transitional/naive B cells [47], was found to be present on the majority of B cells throughout B cell maturation, but is decreased in post-GC B cells (IgD^-CD38^+) and low on plasmablasts (IgD^-CD38^{+++}) (Supporting information, Fig. S2). No differences were found between HC and patients with ME/CFS in the five B cell subsets; we found a significant increase in frequency of BAFF-R in plasmablasts in patients compared to HC ($P < 0{\cdot}01$). However, as the numbers of plasmablasts were low in both groups, the significance of this is questionable.

CD21 (complement receptor, CR2) and CD23 (FcεR, low-affinity receptor for IgE) are both important in B cell antigen presentation [48,49]. Relative frequencies of CD21 and of CD23 in B cell subsets defined by IgD/CD38 are shown in Supporting information, Fig. S3a,b. CD21 was found on most B cell subsets but decreased with differentiation towards the plasmablast stage. CD23 was present in approximately 50% of naive B cells and decreased rapidly with differentiation to memory/plasmablast phenotype. No differences were found between HC and ME/CFS patients for frequency of CD21- and CD23-positive cells. Although there was a trend for a higher percentage of CD21-positive cells in IgD^+CD38^- B cells, but this was not significant ($P = 0{\cdot}09$; Supporting information, Fig. S3a). CD5 was present on most transitional B cells (±70%), but in small numbers on the remaining subsets. CD5 is associated mainly with the early stages of peripheral B cell

Table 3. Percentage of B cells ($CD19^+$) in total lymphocytes and B cell subsets defined by immunoglobulin (Ig)D/CD27 and IgD/CD38 in healthy controls (HC) and myalgic encephalomyelitis/chronic fatigue syndrome (ME/CFS) patients.

	HC (n = 32)	ME/CFS (n = 38)	P-value
Total B cells ($CD19^+$)	4·6 (1·6–13·6)[‡]	4·7 (1·0–13·6)	0·80[†]
B cell subsets using IgD and CD27 (%$CD19^+$ B cells)			
Naive (IgD^+CD27^-)	58·6 (32·2–89·9)	57·1 (21·0–86·3)	0·90
IgD^+ pre-switch (IgD^+CD27^+)	16·6 (3·6–31·1)	19·1 (1·2–32·8)	0·46
Switched memory (IgD^-CD27^+)	18·0 (1·6–39·0)	17·4 (1·2–47·0)	0·40
Double-negative memory (IgD^-CD27^-)	4·6 (1·8–20·1)	4·8 (1·4–14·0)	0·99
B cell subsets using IgD and CD38 (%$CD19^+$ B cells)			
Transitional (IgD^+CD38^{++})	6·7 (1·8–15·0)	6·0 (0·7–6·7)	0·71
Naive (IgD^+CD38^+)	56·2 (34·3–80·20)	54·5 (21·8–77·7)	0·69
IgD^+ memory (IgD^+CD38^-)	11·6 (2·7–22·0)	13·9 (4·8–30·1)	0·12
Post-GC memory (IgD^-CD38^+)	12·1 (3·7–32·2)	9·5 (2·3–26·0)	0·08
Double-negative memory (IgD^-CD38^-)	7·6 (1·7–20·2)	7·6 (1·6–22·9)	0·63
Plasmablasts (IgD^-CD38^{+++})	1·8 (0·3–4·8)	1·2 (0·3–6·8)	0·60

[‡]Median and (range); [†]Mann–Whitney U-test was used to compare values of B cells and their subsets in HC and ME/CFS patients. GC = germinal centre.

development [50]. No differences were found between HC and ME/CFS patients (Supporting information, Fig. S4). IgM is the first immunoglobulin expressed on the surface of B cells (transitional and naive B cells), and is secreted by IgD^+ and IgM-only memory B cells [42]. Membrane IgM was therefore confined largely to naive and IgD^+ memory B cells, with no differences in percentages of IgM-positive cells in any subsets between HC and ME/CFS patients (Supporting information, Fig. S5). Further, no increased percentage or expression (mean fluorescence intensity; MFI) of any additional markers (data not shown) was found in total $CD19^+$ B cells except for CD24.

Expression of CD24 in B cell subsets

CD24 was the only additional marker that showed significant differences in total B cells ($CD19^+$) in ME/CFS patients when compared with HC (Fig. 3). After gating for $CD24^+$ cells in total $CD19^+$ B cells (Fig. 3a–c), both percentage and expression (MFI) of CD24 on total $CD19^+$ B cells [Fig. 3e(a) and (b)] were significantly higher in ME/CFS patients than HC ($P < 0·01$ and $P = 0·03$, respectively). CD24 is often used to characterize transitional B cells ($CD24^{++}CD38^{++}$), as shown in Fig. 3D, and here the expression of CD24 was also found to be significantly higher on the patient's B cells (Fig. 3F; $P = 0·03$).

Analyses of the percentage $CD19^+$ B cells positive for CD24 and relative level of expression (MFI) within all subsets, as defined by IgD/CD38, are shown in Fig. 4. Gating strategy and an example for post-GC B cells selected from total $CD19^+$ B cells are shown in Fig. 4a. In Fig. 4b–g the percentages (a) and MFI (b) of CD24 on all five B cell subsets and plasmablasts are shown. There was a significantly increased percentage and MFI of CD24 associated predominantly with subsets co-expressing IgD: namely, transitional B cells [Fig. 4b(a) and b(b)], naive B cells [Fig. 4c(a) and c(b)] and IgD^+ memory B cells [Fig. 4d(a) and d(b)].

As shown in Table 3, there was no difference in the percentage of transitional B cells between ME/CFS patients and HC using IgD/CD38 classification. Although we have found CD24 to be significantly different in ME/CFS patients from HC, these observations require confirmation in extended or alternative cohorts.

Relationships with disease duration

The frequencies of classical B cell subsets and of CD38 (negative, positive and high positive) and of CD27 (negative and positive) on B cells were analysed in relation to age, sex and disease duration. There was a weak negative correlation between percentage CD38 negative B cells and disease duration ($r^2 = 0·15$; $P = 0·02$) (data not shown). CD21 was then used to follow differentiation stages within CD38-negative B cells.

In combination with CD19, the CD19–CD21 complex is an essential B cell co-receptor that functions synergistically to enhance signalling through the B cell antigen receptor in response to T cell-dependent, complement-tagged antigens [51]. By using CD21 along with CD38, the distribution was gated from B cells and representative plots of a HC and a patient are shown in Fig. 5. There was a trend towards an increase in the $CD21^+CD38^-$ B cell population in ME/CFS patients, but this was not significant (data not shown). To compare the relative frequencies of the $CD21^+CD38^-$ population between HC and ME/CFS patients, a cumulative distribution function (CDF) was performed (Fig. 6a). In Table 4, tertiles of the distribution of HC and ME/CFS patients are shown along with odds ratios (95% CI) and P-values. Comparing the groups by tertile, there was no difference in risk between the first and second tertile, but there was a 3·5-fold increase in the odds of ME/CFS

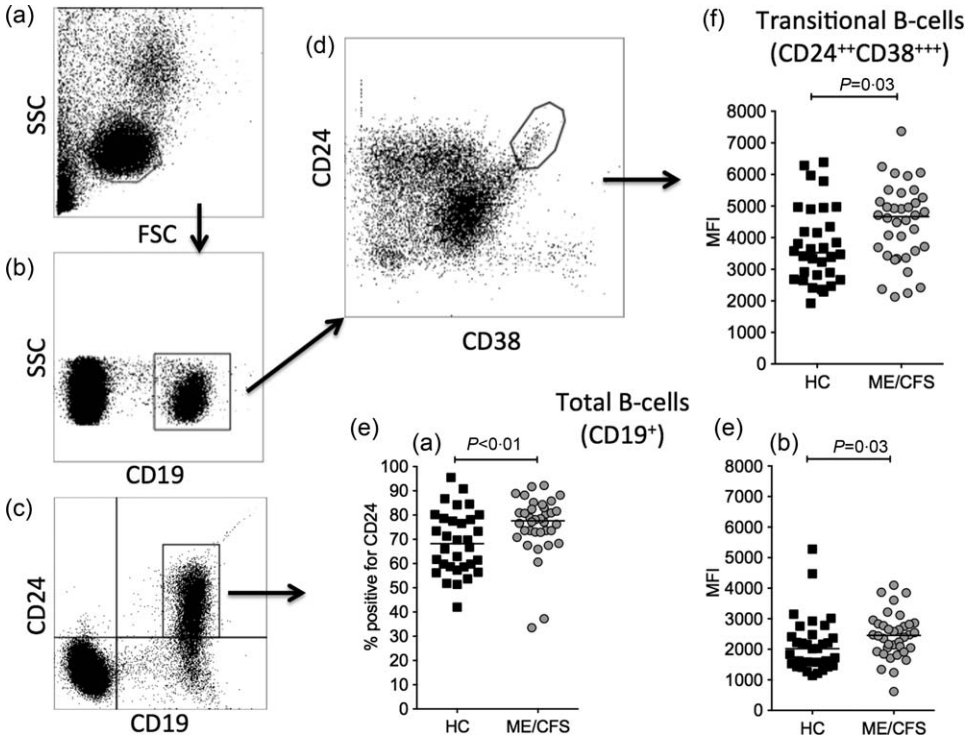

Fig. 3. Frequency and expression of CD24 on B cells in patients with myalgic encephalomyelitis/chronic fatigue syndrome (ME/CFS) ($n = 36$) and age- and sex-matched healthy controls (HC) ($n = 32$). Representative plots (using whole blood from a HC) showing (a) lymphocytes selected based on side-scatter *versus* forward-scatter in which (b) B cells expressing CD19 were then selected. (c) CD24 expressing $CD19^+$ B cells were selected and (d) $CD19^+$ B cells were plotted for CD24 and CD38 to identify transitional B cells ($CD24^{++}CD38^{++}$). In (e), part (a) frequency (%) and in (e) part (b) expression mean fluorescence intensity (MFI) of $CD24^+$ B cells on $CD19^+$ B cells are shown. In (f) expression (MFI) of CD24 on $CD24^{++}CD38^{++}$ transitional B cells is shown; each symbol represents one individual: bar represents median and *P*-values are shown (Mann–Whitney *U*-test).

patients having levels of $CD21^+CD38^-$ B cells of $> 20\%$ ($P = 0{\cdot}05$). When the top tertile was compared to the bottom two tertiles combined ($< 20\%$ $CD21^+CD38^-$) the odds ratio was 3·27 (1·15–10·46; $P = 0{\cdot}03$). Finally, a weak association between disease duration and $CD21^+CD38^-$ was found (Fig. 6b).

Discussion

B cells play an important role in adaptive immunity, primarily by producing antibodies. They are key players in a wide range of immunological diseases, ranging from diminished B cell function (primary or secondary immunodeficiences), B cell transformation (leukaemia, lymphoma) and production of autoantibodies (rheumatoid arthritis and myasthenia gravis). In ME/CFS evidence for B cell dysfunction related to autoimmunity has been limited; however, an increased incididence of B cell lymphoma (mainly marginal zone stage) has been associated with previous history of ME/CFS [52]. In this study we found that serum total IgG levels were elevated in some patients, as is often found associated with autoimmunity [53–55], but this has not been reported in other cohorts.

The most convincing evidence for B cell involvement in ME/CFS has been from a double-blind placebo-controlled clinical trial, where 10 of 15 (67%) of ME/CFS patients receiving the B cell-depleting agent rituximab showed an improvement in symptoms of fatigue, cognition, pain and wellbeing compared to the placebo group (two of 15; 13%) [28]. Similar findings were found after maintenance treatment with rituximab [28,29]. It is unclear whether response to rituximab implicated a direct role for B cells through direct interaction with other immune cells or via B cell products such as antibodies, soluble factors such as cytokines or as a reservoir of B lymphotrophic viruses such as EBV. Rituximab is highly effective in the treatment of CD20-expressing lymphomas and has been used to good clinical effect in autoimmune diseases associated with proven (or suspected) pathogenic autoantibodies, for example by their formation of immune complexes (rheumatoid arthritis and systemic lupus erythematosus) or by autoantibodies binding directly to cell surface receptors, for example acetylcholine receptors (myasthenia gravis) [56,57]. Whether it is beneficial in ME/CFS by removing as-yet unidentified autoantibodies, for example the recently described anti-muscarinic receptor antibodies [34] or by other means, is not yet known.

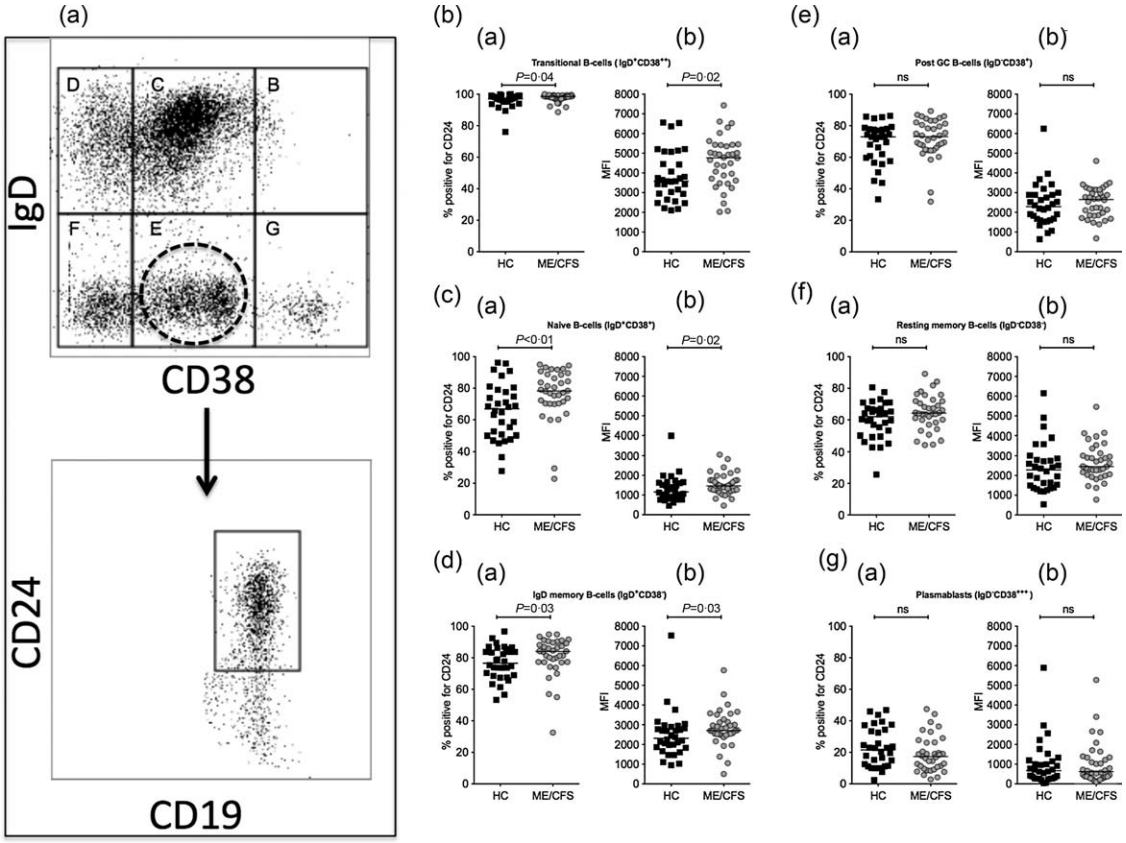

Fig. 4. Frequency and expression of CD24 on B cell subsets in patients with myalgic encephalomyelitis/chronic fatigue syndrome (ME/CFS) (n = 36) and age- and sex-matched healthy controls (HC) (n = 32). (a) Gating strategies used to identify six B cell subsets as defined by immunoglobulin (Ig)D/CD38 and then selected for CD24 are shown (representative plots from a HC). (b–g) For each subset CD24 (a) frequency (%) and (b) expression [mean fluorescence intensity (MFI)] are shown. (b) Transitional B cells; (c) naive B cells; (d) IgD^+ memory B cells; (e) post-germinal centre (GC) B cells; (f) resting memory B cells; and (g) plasmablasts. Each symbol represents one individual: bar represents median and P-values are shown (Mann–Whitney U-test; n.s. = not significant).

Previous studies exploring B cell phenotypes in ME/CFS patients have not shown consistent differences when compared with HC [38–40,58]. Using the classicial B cell markers IgD, CD27 and CD38 to delineate B cell subsets in ME/CFS patients, we did not find a difference (%CD19 and MFI) when compared with HC, confirming studies by Curriu *et al.* In addition, the frequency and expression of BAFF-R, CD5, CD23 and IgM within IgD/CD38-defined populations were also found to be similar to HC. However, we found an increase in both frequency and expression of CD24 on total B cells ($CD19^+$), which was confined to subsets positive for IgD (associated with early B cell subsets plus IgD^+ memory). The MFI of CD24 on transitional B cells, defined by CD24/CD38, was also increased relative to HC, but frequencies were similar. CD24 is a glycoprotein adhesion molecule expressed on the surface of most B cells and differentiating neuroblasts [59]. It is used most extensively as a marker for transitional B cells, and is also expressed on metabolically active naive B cells and, to a lesser extent, in terminally differentiated cells such as memory B cells and plasmablasts. Interestingly, CD24-deficiency in CD24 knock-out mice resulted in the reduction in late pre-B and immature B cell populations in the bone marrow, but the functional consequences of increased expression have not been studied [60–62]. CD24 is highly polymorphic in humans, with some polymorphisms associated with increased risk and progression of autoimmune diseases including multiple sclerosis, rheumatoid arthritis, and systemic lupus erythematous [63–68]. CD24 can also be over-expressed in many cancers, including B cell lymphomas, and appears oncogenic [59]. So far, no single nucleotide polymorphisms (SNPs) or polymorphisms in CD24 have been related to ME/CFS. A study by Fujimoto *et al.* found that cross-linking CD24 induces apoptosis in Burkitt's lymphoma cells, which share similar characteristics with GC B cells [69]. The relative strength of antigen binding to the B cell receptor (BCR) largely determines B cell survival, together with context-dependent signals from T cells and cytokines, but other stimuli mediated by co-stimulatory molecules such as CD24 work in synergy with the BCR, perhaps changing the stringency of autoreactive B cell selection and expansion.

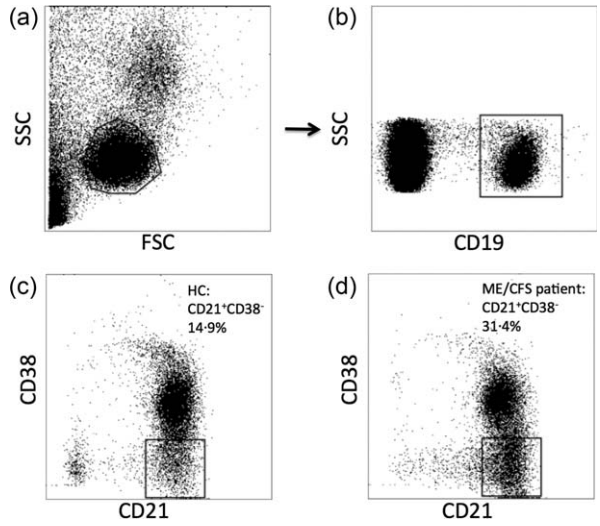

Fig. 5. Distribution of $CD21^+CD38^-$ B cells in healthy controls (HC) and myalgic encephalomyelitis/chronic fatigue syndrome (ME/CFS) patients. Representative plots (using whole blood from a HC) showing (a) lymphocytes selected based on side-scatter *versus* forward-scatter and (b) B cells expressing CD19. In (c) and (d) relative expression of $CD21^+CD38^-$ on B cells within the $CD19^+$ gate are shown for a HC and a ME/CFS patient, respectively.

We also found differences from HC in the percentages of $CD38^-$ memory B cells, which were positive for CD21. The frequencies of these cells were predominantly $CD27^+$ and associated weakly with disease duration. A high percentage

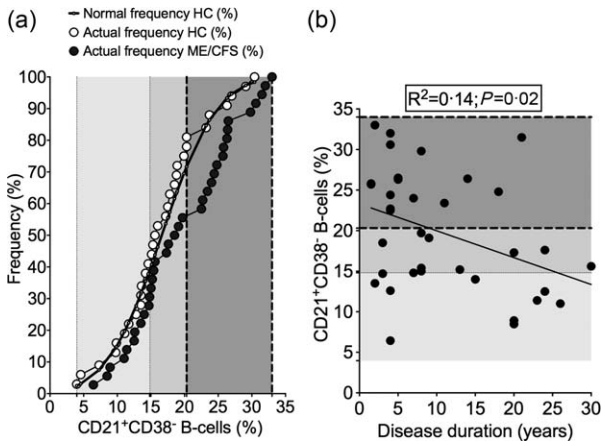

Fig. 6. Cumulative distribution function and association with disease duration of $CD21^+CD38^-$ B cells. (a) The calculated (normal) frequency of $CD21^+CD38^-$ B cells in healthy controls (HC) and the actual frequencies of $CD21^+CD38^-$ B cells in HC and myalgic encephalomyelitis/chronic fatigue syndrome (ME/CFS) patients were plotted in a cumulative distribution function graph. Distribution is divided into three tertiles, as shown by the same shading in each graph. (b) The percentage of $CD21^+CD38^-$ B cells in ME/CFS patients were plotted against disease duration. Each symbol represents one individual: dashed lines represent cut-offs for tertiles. Statistical significance was calculated using linear regression and Pearson's correlation coefficient is shown.

Table 4. Comparison of frequencies of $\%CD21^+CD38^-$ B cells in healthy controls (HC) and myalgic encephalomyelitis/chronic fatigue syndrome (ME/CFS) patients.

Tertile	HC (n = 32)	ME/CFS (n = 38)	Odds ratio (95% CI)	P-value
1 (<14·8)	40·6	27·8	1·00	–
2 (14·8–20·3)	40·6	27·8	1·00 (0·31–3·21)	1·00
3 (>20·3)	18·8	44·4	3·47 (0·99–12·09)	0·05*
1 + 2 *versus* 3 (≤20·3 *versus* >20·3)			3·47 (1·15–10·46)	0·03*

*Significant ($P < 0·05$). CI = confidence interval.

of this memory B cell population was also associated with the presence of ME/CFS, with a significantly increased relative risk. A study in macaque monkeys has shown that $CD21^+CD27^+$ B cells produce antibodies by polyclonal activation [pokeweed mitogen (PWM), protein A and CpG2006] without T cell help and also that this memory B cell population was able to proliferate at a higher rate than other B cell subpopulations [70]. Further studies of functional properties in ME/CFS need to be investigated to determine the importance or otherwise of these findings.

There is a general consensus that the pathophysiology of ME/CFS involves the central nervous system with changes in neural signalling, which persist throughout the course of the illness. Metabolic abnormalities such as changes in mitochondrial function in muscle and other tissues are also well described in these patients [71–73]. Immune system involvement in the periphery in the form of autoantibodies or cytokines could therefore underlie chronic changes to the central nervous system by dysregulating neural pathways.

Recently, Hornig *et al.* reported disturbed immune signatures in the cerebrospinal fluid of ME/CFS cases consistent with immune activation in the central nervous system, and a shift towards an allergic or T helper type 2 pattern [20]. The same group reported that there was a dissociation of intercytokine regulatory networks in ME/CFS cases where CD40L was found to be decreased and interferon (IFN)-α increased in patients with a disease duration of 3 years or less compared to those with > 3 years history and also with HC [21]. These findings were suggested to be the consequences of immune triggering following an infection.

An important difference between ME/CFS and established diseases is the absence of clear symptom-based 'biomarkers' which can be compared with control groups. Because ME/CFS is highly heterogeneous, it might therefore be more productive to identify possible subgroups of patients both for the purpose of research and to target therapeutic interventions.

Conclusion

ME/CFS is a complex condition, but this preliminary study using additional combinations of B cell surface markers,

not used before in ME/CFS phenotyping, confirmed the little overal difference in B cell subsets found in earlier studies, stating that our most important findings were found by using extended markers. We have tentatively identified subgroups of patients on the basis of B cell phenotype and disease duration. Retention of CD24 and CD21 within B cell subsets may have implications for changes in thresholds for B cell differentiation. If confirmed in other patient cohorts, these results may provide a possible platform for functional studies and to follow disease course over therapy.

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Disclosure

The authors have no disclosures to declare.

Author contributions

F. M. performed all the experiments under the supervision of G. C. F. M. and A. S. designed the patient protocols. M. J. L. and V. R. contributed to discussions of results. A. B., S. B., M. J. L., V. R. and A. S. reviewed the manuscript. F. M. and G. C. wrote the manuscript. Clinical assessments and recruitment of patients was by A. B. and S. B. All authors also had access to raw data.

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Supporting information

Additional Supporting information may be found in the online version of this article at the publisher's web-site:

Fig. S1. (a) Frequency (%) of CD27 on $CD19^+$ B cell subsets from healthy controls (HC) ($n = 31$) and myalgic encephalomyelitis/chronic fatigue syndrome (ME/CFS) patients ($n = 35$) in subsets defined by immunoglobulin (Ig)D/CD38 and (b) frequency of CD27 on $CD21^+CD38^-$ B cells (box and whiskers). Each symbol represents one individual: bars represent median values.

Fig. S2. Frequency (%) of B cell activating factor (BAFF)-R on $CD19^+$ B cell subsets in healthy controls (HC) ($n = 32$) and myalgic encephalomyelitis/chronic fatigue syndrome (ME/CFS) patients ($n = 36$) as defined by relative expression of immunoglobulin (Ig)D/CD38. Each symbol represents one individual: bars represent median values.

Fig. S3. (a) Frequency (%) of CD21 and (b) CD23 on $CD19^+$ B cell subsets in healthy controls (HC) ($n = 32$) and myalgic encephalomyelitis/chronic fatigue syndrome (ME/CFS) patients ($n = 36$) as defined by relative expression of immunoglobulin (Ig)D/CD38. Each symbol represents one individual: bars represent median values.

Fig. S4. Frequency (%) of CD5 on $CD19^+$ B cell subsets in healthy controls (HC) ($n = 32$) and myalgic encephalomyelitis/chronic fatigue syndrome (ME/CFS) patients ($n = 36$) as defined by relative expression of immunoglobulin (Ig)D/CD38. Each symbol represents one individual: bars represent median values.

Fig. S5. Frequency (%) of immunoglobulin (Ig)M on $CD19^+$ B cell subsets in healthy controls (HC) ($n = 27$) and myalgic encephalomyelitis/chronic fatigue syndrome (ME/CFS) patients ($n = 32$) as defined by relative expression of IgD/CD38. Each symbol represents one individual: bars represent median values.